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THE FUNCTION OF FRESH AND CRYOPRESERVED MONOCYTES ASSESSED BY RELEASE OF 51-CR RADIOACTIVITY FROM HUMAN RED BLOOD CELLS COATED WITH ANTIBODY

BY

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In the first series of experiments, the functional activity of the monocytes appeared to be maintained during cryopreservation: the functional activity was 40% in the fresh samples and 45% following cryopreservation. However, the fresh samples were not assayed along with the cryopreserved samples, and there was a high degree of variability associated with the assay.

In the second series of experiments, the cryopreserved mononuclear cells were assayed along with the fresh samples. The 22% functional activity observed in the cryopreserved samples was significantly lower than the 32% functional activity observed in the fresh samples. However, there w as no correlation between the length of frozen storage and the functional activity.

In vitro viability was assessed by testing membrane integrity using fluorescein diacetate and ethidium bromide and showed that the viability of the cryopreserved mononuclear cells was maintained at 90%.

Human peripheral blood mononuclear cells can be cryopreserved at -80 C for as long as 2.5 years with only a slight loss of functional activity and in vitro viability.

ABSTRACT

Human peripheral blood mononuclear cells isolated from plateletpheresis were cryopreserved at -80 C with 10% DMSO for as long as 2.5 years. Monocyte functional activity was assessed before and after cryopreservation by the release of radioactivity during incubation with 51-Cr labeled red blood cells. The adherent population of mononuclear cells was isolated to increase the proportion of monocytes in the mononuclear cell samples before testing for functional activity.

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INTRODUCTION

Human peripheral blood mononuclear cells collected during plateletpheresis have been shown to exhibit in vitro recovery values of 80% following freezing, thawing, and washing (1).

Two separate studies from this laboratory are reported here. In our first study, peripheral blood monocytes were assayed for functional activity before and after cryopreservation. The second study was designed to assess the effects of long-term frozen storage on the functional activity and viability of the mononuclear cells. The mononuclear cells were isolated from peripheral blood during plateletpheresis and were tested for monocyte functional activity and membrane integrity before cryopreservation and after frozen storage for as long as 2.5 years.

Monocyte-rich mononuclear cells were prepared by isolating the adherent populations of fresh and previously frozen mononuclear cells. The functional activity of the mononuclear cells was assessed by measuring the release of radioactivity during incubation with antibody coated ^{51}CR-labeled human red blood cells. Viability was evaluated by testing the membrane integrity of the mononuclear cells before and after isolation of the adherent cells.

METHODS

Isolation of peripheral blood mononuclear cells

The mononuclear cells were isolated from the plateletpheresis residue by the Ficoll-Hypaque density (1.077 g/ml) gradient centrifugation procedure using a plastic bag system (ETHOX corp.) (1,2). The cells were washed once with 0.9% sodium chloride and were resuspended in approximately 85 ml autologous ACD plasma.

Cryopreservation of mononuclear cells

A 3 ml aliquot of the fresh mononuclear cell suspension was used for the functional assay, viability testing, and size distribution measurements. Approximately 40 ml of the cell suspension, containing approximately 6 X 10^8 cells, was frozen at 2 to 4 C per minute with 10% DMSO in autologous plasma in a PL146 polyvinylchloride plastic bag (Fenwal Laboratories, Deerfield, Il) at -80 C and stored for as long as 2.5 years. The frozen mononuclear cells were thawed and washed in a solution of 0.9 g% sodium chloride-0.2 g% glucose-40 mg% phosphorus, pH 5, and then resuspended in approximately 50 ml autologous plasma.

Monocyte Functional Assay

The monocyte functional activity of the mononuclear cells was assayed using a modification of the procedure described by Kurlander and Rosse (3).

A. Isolation of adherent mononuclear cells (adMNC):

A 1 ml volume of a fresh or previously frozen mononuclear cell suspension was washed three times with a 5 ml volume of RPMI 1640 medium containing calcium and magnesium (Gibco, Grand Island, NY) supplemented with Hepes (Calbiochem, San Diego, Ca.), heat-inactivated fetal calf serum (Hyclone, Logan, Ut), and a volume of acid-citrate-dextrose solution (ACD) (Squibb Diagnostics, New Brunswick, NJ) equivalent to 10% of the volume of medium. Each of the solutions was filtered through a 0.45 micron and a 0.22 micron filter (Nalgene) prior to use. The mononuclear cells were resuspended in the supplemented RPMI medium without ACD to a concentration of 5×10^6 cells per ml in approximately 20 ml of solution.

Monocyte-rich cells were prepared by adding 10 ml of the mononuclear cell suspension (containing 5×10^7 mononuclear cells) to a plastic petri dish and allowing the mononuclear cells to adhere to the petri dish during 1½ hours of incubation at 37C in a 5% CO2 incubator and then decanting the non-adherent cells from the petri dish. The petri dish was rinsed three times with the supplemented RPMI medium without ACD, and the adherent cells were gently scraped from the petri dish into a 15 ml test tube and centrifuged. The supernatant solution was removed, and the cells were resuspended in the medium without ACD to a concentration of 5×10^6 cells per ml in a final volume which ranged from 0.5 to 1.0 ml. This final monocyte-rich

cell preparation is referred to as adherent mononuclear cells (adMNC). The percentages of monocytes in the sample applied to the petri dish and in the sample collected from the petri dish as adherent cells were determined from volume distribution measurements using a Coulter Counter model C1000 (Coulter Electronics, Hialeah, Fla.).

B. Preparation of 51CR-labeled antibody-coated red blood cells:

Human Rh positive red blood cells from a 100 ul blood sample were washed 3 times with 0.9% saline and resuspended to a hematocrit of >75%. The red cells were incubated at 37C with 30 uCi/ml of disodium chromate (^{51}CR) (Squibb diagnostics, Princeton, N.J.) for 1 hour, then washed 3 times in 0.9% saline, separated into two equal aliquots, and centrifuged. One aliquot of the red blood cells was incubated at 37C for 1 hour with 500 ul of a dilution of a serum containing anti-D antibody (1:100 Rhesonative, Kabi Vitrum AB, Stockholm, Sweden); and the control was incubated for 1 hour with 500 ul 0.9% saline. The cells were washed 3 times with supplemented RPMI medium, and resuspended to 10 X 10^7 cells / ml.

C. Assay procedure:

The anti-D coated 51Cr-labeled red cells with or without adherent mononuclear cells (adMNC) and the non-antibody coated ^{51}Cr-labeled red cells with or without adMNC were incubated in a 5% CO_2 incubator at 37C for 24 hours. A

volume of 0.05 ml of a 1 X 10^7 concentration of red cells was combined with a 0.10 ml volume of a 5 X 10^6 concentration of adMNC. The number of red cells and adMNC were 5 X 10^5 each in the final 0.15 ml volume. After incubation, the 4 samples were centrifuged and the cells were pelleted. The supernatants were removed and counted for ^{51}Cr activity in a well-type scintillation counter (TM Analytical, Elk Grove Village, Il). A sample of non-centrifuged ^{51}Cr labeled red cells without adMNC was also counted for radioactivity to determine the 100% radioactivity value. The percentage of red cells lysed as a result of monocyte activity was calculated from the radioactive ^{51}Cr counts in the supernatant of the red cells with monocytes minus the counts in the supernatant of the red cells without monocytes divided by the total radioactivity. The percentage is reported as the functional activity of the monocytes. The percentage of ^{51}Cr released from the red cells without monocytes is reported as spontaneous hemolysis. An arbitrary level of less than 15% spontaneous hemolysis was considered acceptable for the assay.

In vitro viability testing

The mononuclear cells were tested for membrane integrity (3) prior to and following recovery of the adherent cells from the petri dishes. Both the fresh and previously frozen cells were incubated with a fluorescein

diacetate and ethidium bromide mixture and viewed for fluorescence using a fluorescence microscope.

Study 1: Functional activity of fresh and previously frozen mononuclear cells

In the first study, we assayed the functional activity of peripheral blood mononuclear cells before and after cryopreservation at -80C for as long as 126 days. These functional assays were performed on 18 freshly isolated mononuclear cell samples. In this study the fetal calf serum used was not heat-inactivated; in the subsequent experiments it was.

The correlation between the ratio of red cells to adherent mononuclear cells and the functional assay was investigated using heat-inactivated fetal calf serum. The ratios ranged from 1 red cell for every 4 adherent mononuclear cells to 4 red cells for each adherent mononuclear cell in a constant incubation volume of 0.15 ml. The percentage of ^{51}CR released from antibody coated red cells was recorded as monocyte functional activity. A total of nine mononuclear cell preparations were studied: 6 of the 9 were studied before cryopreservation and 8 of the nine were studied after cryopreservation.

In vitro viability and functional assays using heat-inactivated fetal calf serum were performed on 11 fresh adherent mononuclear cell samples, and on 13 adherent previously frozen adherent mononuclear cell samples, 7 of which were evaluated both before and after cryopreservation.

Study 2: The effects of donor variability and of -80C storage of the mononuclear cells on monocyte function

In this study, fresh mononuclear cell samples were assayed along with previously frozen samples, to determine the variability in the assay.

To assess the effect of the variability in the functional assay, seven blood samples were obtained from one donor and assayed over a 295 day period, and 6 blood samples from a second donor were assayed over a 267 day period. In addition, functional assays were done on a fresh and previously frozen blood sample obtained from the same donor: on the day that the frozen sample was thawed, washed and assayed, a fresh blood sample was obtained from the same donor for testing.

The mononuclear cells were stored at -80C from 79 to 936 days. The in vitro viability testing, and the functional assay of the adherent mononuclear cells were performed before and after cryopreservation. Thirty five fresh samples and 11 cryopreserved samples were studied.

RESULTS

A schematic of the functional assay is depicted in Figure 1.

Spontaneous hemolysis during the functional assay

The 51Cr radioactivity released from the antibody-coated 51Cr-labeled red blood cells without monocytes is reported as spontaneous hemolysis. Failure to properly heat-inactivate the complement in the fetal calf serum caused increased spontaneous hemolysis of 51.2% in the 18 studies reported in Table 1. The temperature of the water bath for heat-inactivation was 49C instead of the necessary 56C. In subsequent studies in which the fetal calf serum was heat-treated at 56C, spontaneous hemolysis of >15% occured on two occasions.

Ratio of ^{51}CR red cells to adherent mononuclear cells used in the functional assay

In the functional assay, a correlation was seen between the ratio of 51Cr-labeled antibody coated red blood cell to adMNC and the release of 51Cr from the antibody coated red blood cells (Table 2, Figure 2). The data show that a ratio of 0.5 antibody-coated red blood cells to 1.0 adMNC produced the highest percentage of lysis of 51Cr-labeled antibody-coated red blood cells (Figure 2). Frozen adMNC exhibited greater lysis of the antibody-coated red blood cells than did fresh adMNC (Figure 2). The ratio of one adMNC to one

51Cr-labeled antibody-coated red blood cells produced approximately 40% lysis, and this was the ratio used in the assay.

Percentage of monocytes in the adMNC cell samples

The percentage of fresh adMNC identified as monocytes by volume distribution ranged from 14 to 90%, with a mean value of 61% (Table 4). The percentage of previously frozen adMNC identified as monocytes ranged from 13 to 67%, with a mean value of 42% . All of the adherent cells were removed from the petri dish, indicating that retention of adherent cells on the petri dish was not responsible for the variability in the percentages of monocytes in the adMNC samples.

Functional activity in the first study

The functional assay showed no differeces between fresh and previously frozen mononuclear cells in the first study (Tables 3,4). The mean functional activity of the fresh adMNC was 40%, with a range of 10 to 61%, that of the previously frozen mononuclear cells was similar, at 45%, with a range of 26 to 58%.

Viability *in vitro* and functional activity in the second study

In vitro viability and functional activity of_fresh mononuclear cells in the second study are reported in Table 5. The values for previously frozen mononuclear cells are

reported in Table 6. Tests of membrane integrity of the adMNC using fluorescein diacetate and ethidium bromide showed only a slight reduction in viability, from 98% before plating to 90% after plating, for the fresh mononuclear cells. The viability values for previously frozen mononuclear cells before and after plating were 90 and 89% respectively. The mean functional activity for the fresh mononuclear cells was 27% with a range of 4 to 44%, and for previously frozen mononuclear cells the mean was 22% with a range of 1 to 36%. The overall values were lower in this study than in the first study (Table 7).

There was no significant correlation between viability and functional activity in either fresh or previously frozen adherent mononuclear cells (Figure 3).

Fresh and cryopreserved monocytes

There was no significant difference in the functional activity of mononuclear cells before (22.4%) and after freezing (20.2%); however, when the previously frozen adMNC were compared to the fresh adMNC, a paired analysis showed a statistically significant difference, ($p=.0141$). The functional activity of previously frozen adMNC was 68% of the fresh adMNC.

The functional assays of fresh and previously frozen adMNC for studies 1 and 2 are summarized in Table 7 and Figure 4.

Effect of the length of frozen storage at -80C

In the first study in which the frozen monocytes were stored at -80C for as long as 126 days (Table 7), the previously frozen adMNC exhibited functional activity similar to that of fresh adMNC.

In the second study in which the frozen monocytes were stored at -80C for as long as 936 days, the previously frozen adMNC exhibited a mean functional activity 32% lower than that of the fresh adMNC (Table 7). The functional activity of the previously frozen adMNC expressed as a percentage of fresh adMNC was not related to the length of frozen storage (Table 8, Figure 5).

Percentage of monocytes in the mononuclear cell samples

The percentages of monocytes identified by volume distribution in the mononuclear cell samples before plating and in the adherent mononuclear cells recovered from the plates for fresh and cryopreserved samples in study 2 are reported in Table 9. For the fresh samples, the mean percentage of monocytes was 25% before plating, and increased significantly ($p<0.001$) to 45% following plating. The values ranged from 10 to 55% before plating and 10 to 73% after plating. The cryopreserved samples contained 28% monocytes before plating and 34% after plating, which was not a significant difference. The percentages of monocytes

measured in the adherent cells after plating were lower in this second study than in the first study.

The relation between the % monocytes in the adMNC sample and the functional assay of the adMNC is shown in figure 6. The analyses of the data for fresh and cryopreserved cells from both studies showed that in the cryopreserved cells in study 2, there was a statistically significant ($p<0.05$) relationship between the percentage of monocytes and the functional activity.

Correction of the functional assay for % viability and % monocytes

When the functional assay was corrected for the percentage of monocytes in the adherent cell sample and for viability, the red cell lysis per viable monocyte ranged from 21 to 259% (Table 10).

Donor variability

The data in Table 11 show that there was considerable variability in the monocyte assay performed on a series of fresh samples from a single donor. The variability within the same donor was similar to that among random donors tested over the same time period (Figure 7).

DISCUSSION

Human blood mononuclear cells isolated from peripheral blood have been cryopreserved, with preservation of membrane integrity and maintenance of pluripotential activity of the mononuclear cells in the CFU-GEMM tissue culture assay (1).

Monocyte functional activity and in vitro viability of peripheral blood mononuclear cells were studied before and after long-term cryopreservation with 10% DMSO.

The adherent population of mononuclear cells was isolated in order to increase the concentration of monocytes in the mononuclear cell samples before testing for functional activity. This procedure increased the percentage of monocytes from 25 to 45% in fresh samples and from 28 to 34% in cryopreserved samples.

The first study established the optimum ratio of red cells to adherent cells at 1 to 1 for the functional assay, and spontaneous hemolysis was controlled by the proper inactivation of complement in the fetal calf serum. However, there was a high degree of variability associated with the assay.

Assays of the mononuclear cells in the first study showed no apparent loss of functional activity during cryopreservation: functional activity was 40% in the fresh samples and 45% in the cryopreserved samples.

In the second study, the functional activity in fresh adherent mononuclear cells was 33% lower overall than in the

first study. The functional assay of the cryopreserved adherent mononuclear cells was 22%; which was significantly lower than the 31% for fresh adherent mononuclear cells. The reduced activity associated with cryopreservation may have been overlooked in the first study because the fresh samples were not assayed along with the cryopreserved samples. The duration of storage at -80C for as long as 2.5 years had no detectable effect on the functional activity of the adherent mononuclear cells.

In the second study, on the day that the previously frozen mononuclear cells were tested, fresh mononucelar cells were collected from the same donor. In some cases, the same donor provided fresh mononuclear cell samples on as many as 7 occasions. The variability in the functional assay was similar whether the fresh mononuclear cells were obtained from a single donor or from several donors.

The membrane integrity was only slightly affected by plating and by cryopreservation. The viability of the fresh mononuclear cells exceeded 94%, with only an 8% loss associated with the plating procedure to collect the adherent cells and a mean loss of 10% viability following cryopreservation.

We observed a correlation between the functional assay and the ratio of red cells to adherent mononuclear cells. The percentage of monocytes in the adherent cell samples could not account for the variability in the functional assay. The percentage of monocytes in the adherent

mononuclear cell samples was determined by the volume distribution of the cells, which may not have been an accurate measurement.

These studies demonstrate that adherent mononuclear cells following cryopreservation at -80C for as long as 2.5 years have functional activity only slightly less than that of fresh adherent mononuclear cells.

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4. Lionetti F.J., Hunt S.M., Lin P.S., Kurtz S.R., Valeri C.R.: II Characteristics of Granulocytes Obtained By Counterflow Centrifugation: Transfusion, 17:465-472, 1977.

FIGURE 1

A schematic describing the monocyte functional assay performed on fresh and frozen adherent mononuclear cells-monocytes.

FIGURE 1

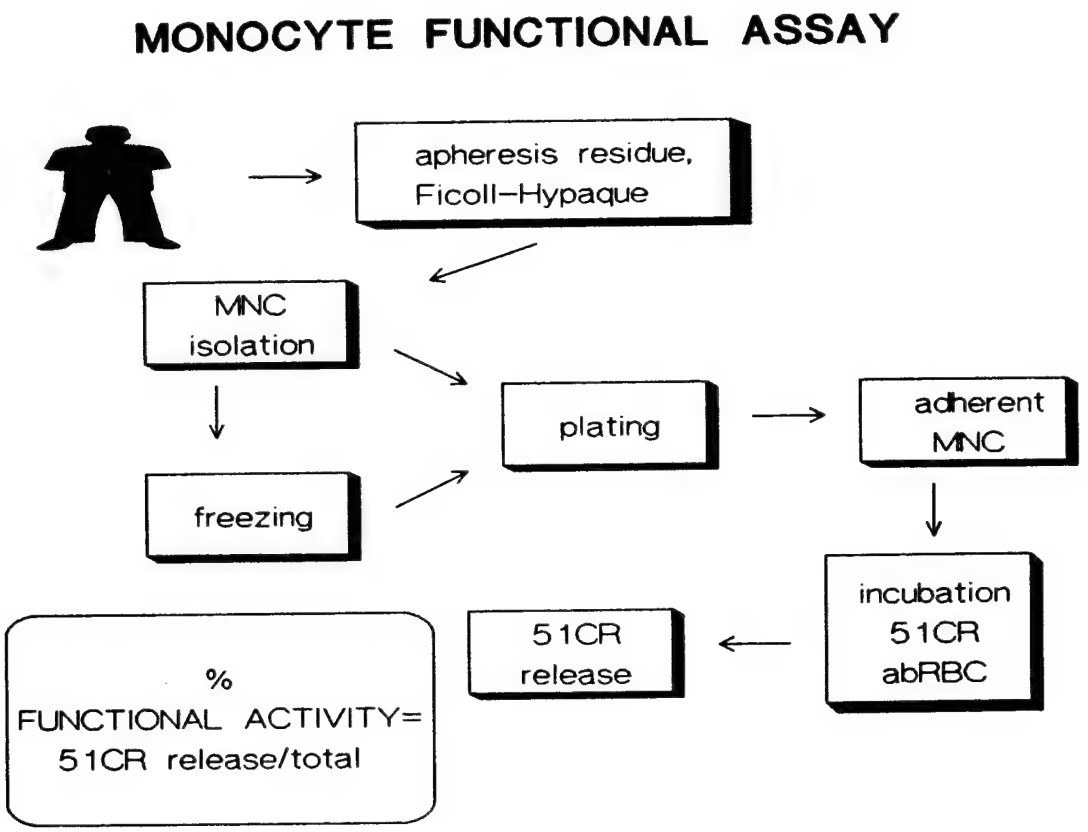
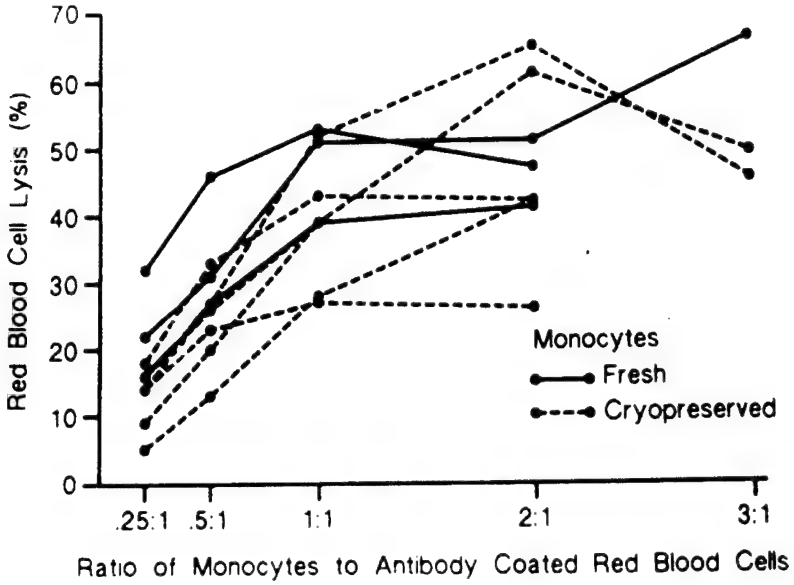

MONOCYTE FUNCTIONAL ASSAY
apheresis residue,
Ficoll-Hypaque
MNC
isolation
freezing
plating
adherent
MNC
incubation
51CR
abRBC
51CR
release
%
FUNCTIONAL ACTIVITY=
51CR release/total

FIGURE 2

The effects of concentrations of fresh or cryopreserved monocytes and of antibody coated red blood cells on fresh and cryopreserved monocyte functional assay.

EFFECT OF CONCENTRATION OF FRESH OR CRYOPRESERVED MONOCYTES ON THE MONOCYTE FUNCTIONAL ASSAY



EFFECT OF CONCENTRATION OF ANTIBODY COATED RED BLOOD CELLS ON THE FRESH OR CRYOPRESERVED MONOCYTE FUNCTIONAL ASSAY

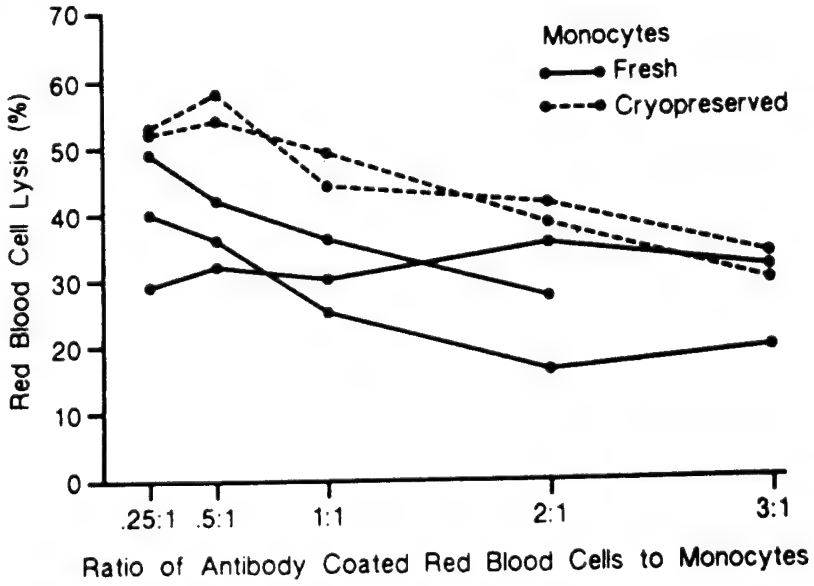


FIGURE 3

The relation between viability testing and functional assay of fresh and cryopreserved adherent mononuclear cell-monocytes.

FIGURE 3

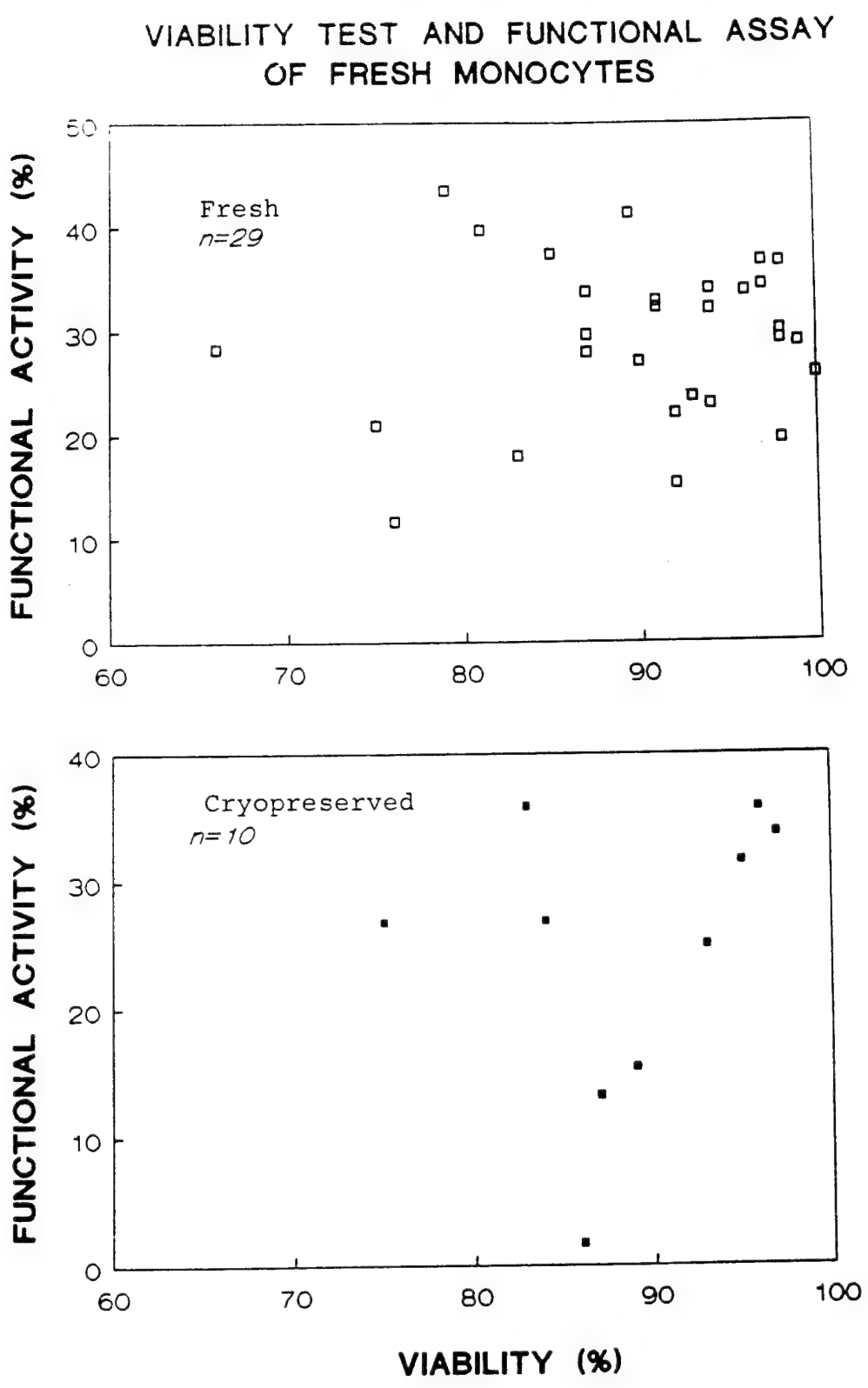
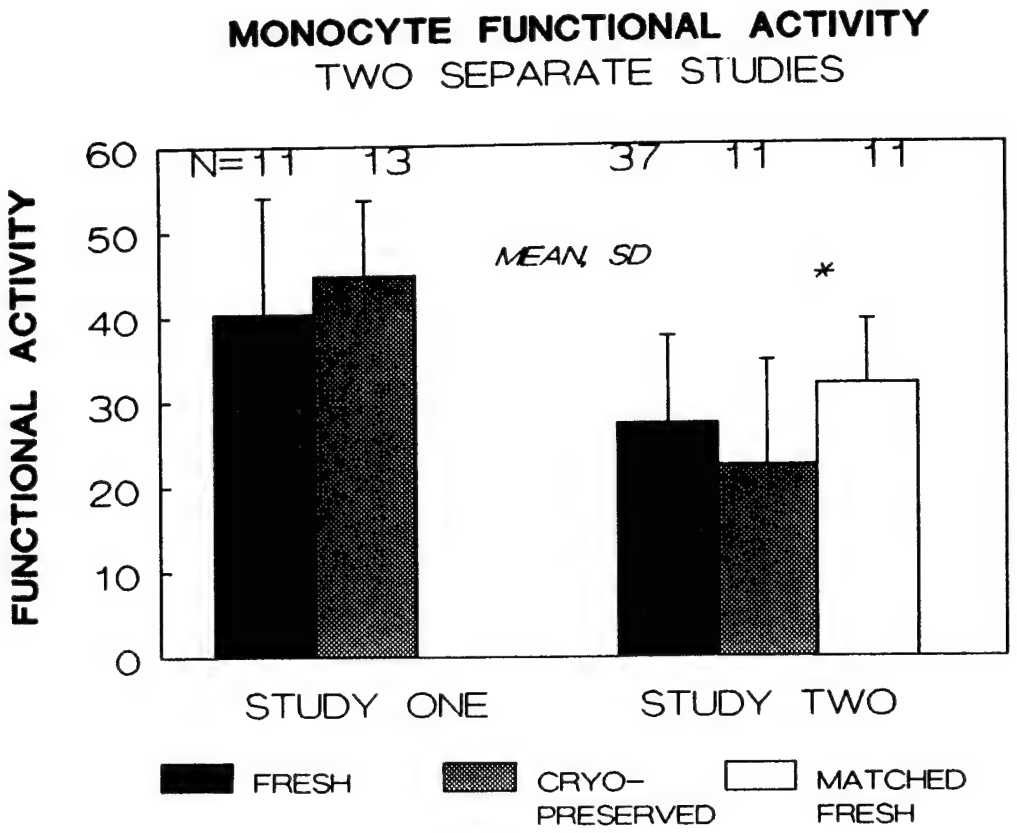

VIABILITY TEST AND FUNCTIONAL ASSAY
OF FRESH MONOCYTES
Fresh
n=29
Cryopreserved
n=10
FUNCTIONAL ACTIVITY (%)
VIABILITY (%)
0
10
20
30
40
50
60
70
80
90
100

FIGURE 4

The functional assay of fresh and cryopreserved adherent mononuclear cells-monocytes done in two separate studies. In study two testing of the fresh monocytes was done on the day of testing of the cryopreserved monocytes. Both fresh and cryopreserved monocytes were obtained from the same donor.

FIGURE 4



*paired t test: $p<0.05$
cryopreserved vs. matched fresh control

FIGURE 5

The effect of storage of the cryopreserved monocytes at -80 C on the functional activity of the monocytes. Both functional activity (%) and the functional activity as a percent of control (%) are reported.

FIGURE 5

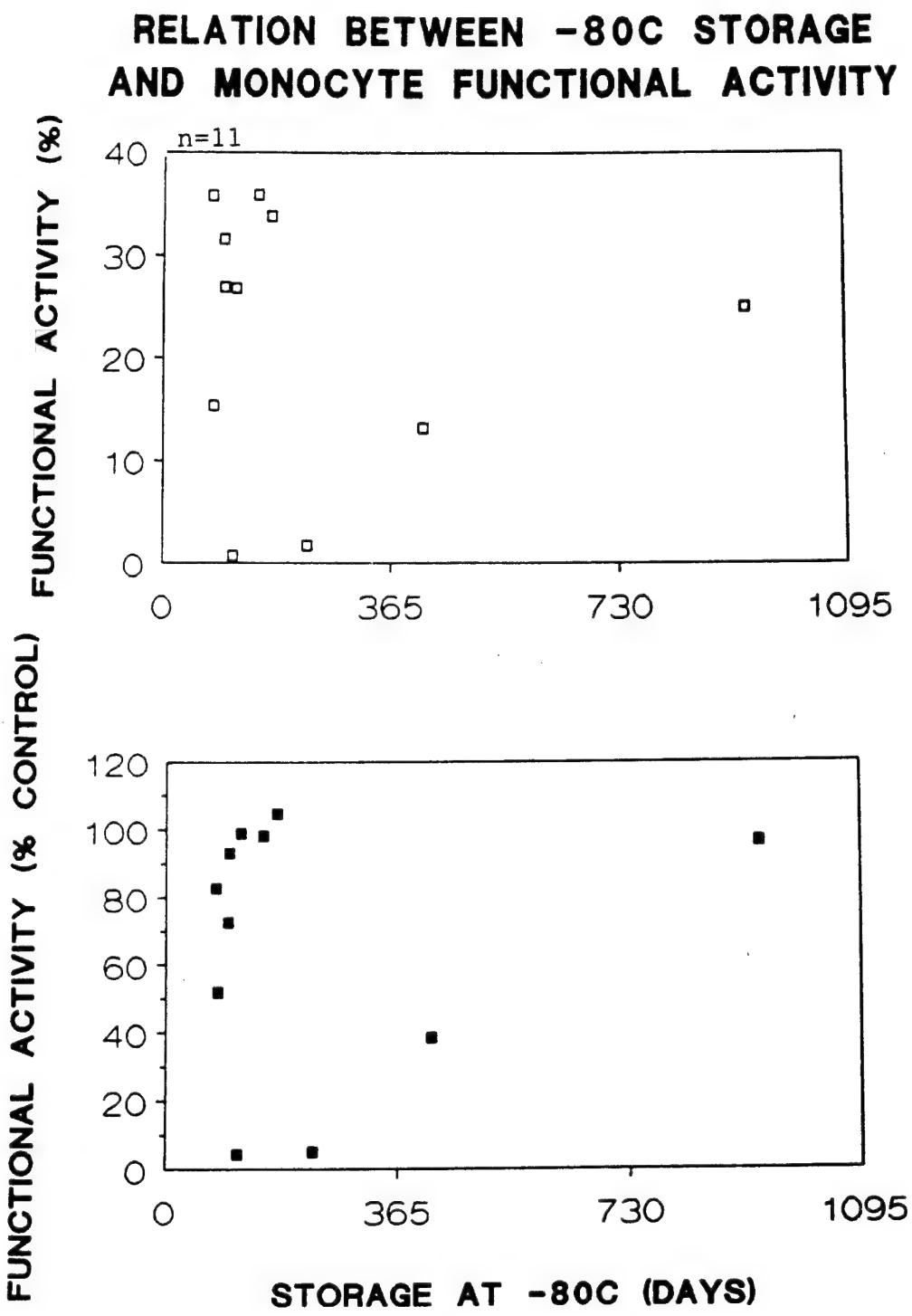
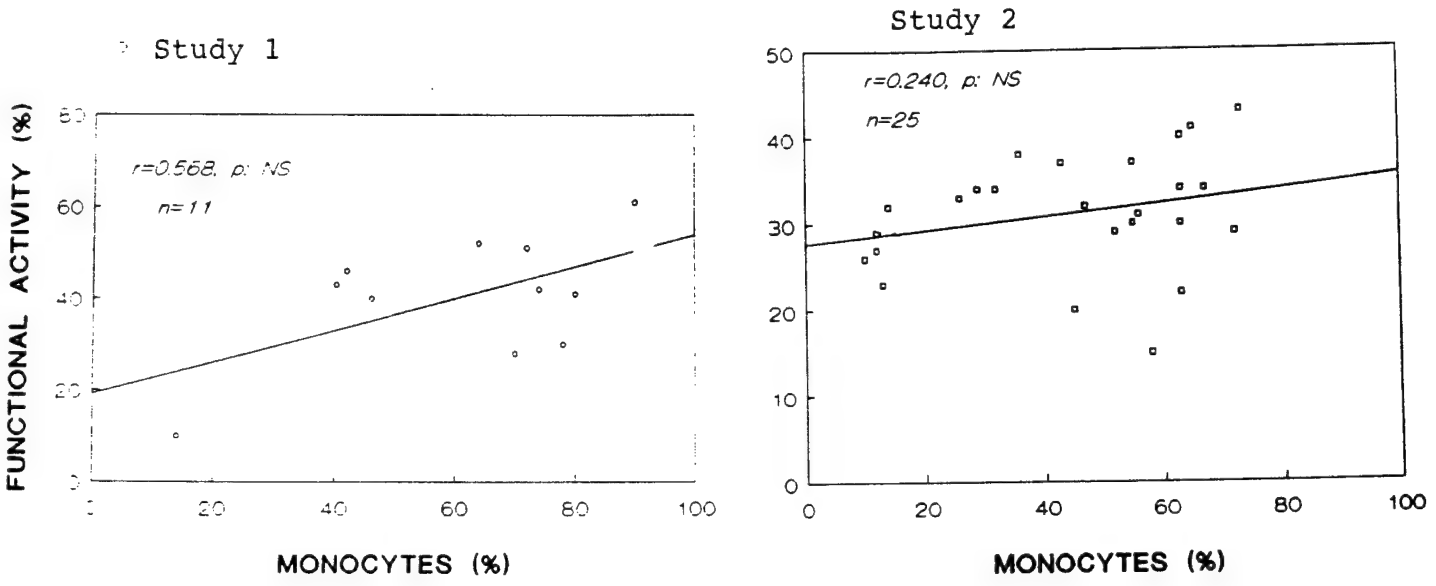

RELATION BETWEEN -80C STORAGE
AND MONOCYTE FUNCTIONAL ACTIVITY
n=11
FUNCTIONAL ACTIVITY (%)
40
30
20
10
0
0
365
730
1095
FUNCTIONAL ACTIVITY (% CONTROL)
120
100
80
60
40
20
0
0
365
730
1095
STORAGE AT -80C (DAYS)

FIGURE 6

The effect of the percentage of monocytes on the functional activity of fresh and cryopreserved adherent mononuclear cells.

FIGURE 6


% MONOCYTES AND FUNCTIONAL ASSAY OF
FRESH ADHERENT MONONUCLEAR CELLS
Study 1
r=0.568, p: NS
n=11
FUNCTIONAL ACTIVITY (%)
MONOCYTES (%)
Study 2
r=0.240, p: NS
n=25
MONOCYTES (%)

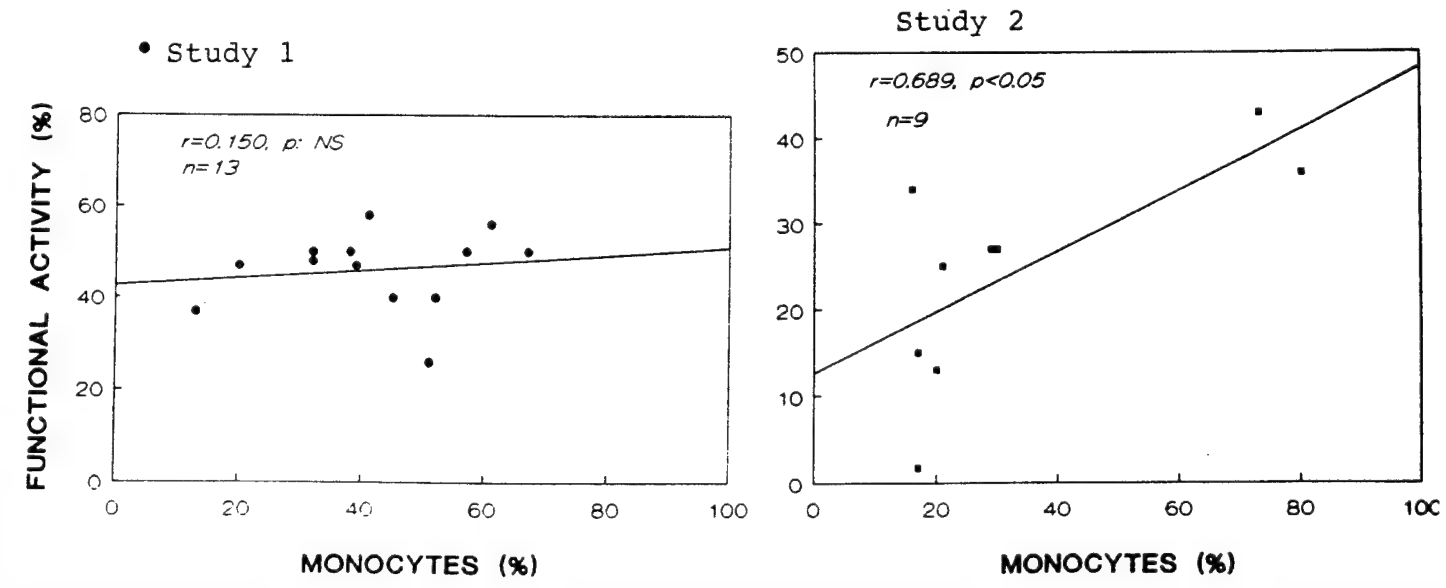

% MONOCYTES AND FUNCTIONAL ASSAY OF
CRYOPRESERVED ADHERENT MONONUCLEAR CELLS
Study 1
r=0.150, p: NS
n=13
FUNCTIONAL ACTIVITY (%)
MONOCYTES (%)
Study 2
r=0.689, p<0.05
n=9
MONOCYTES (%)

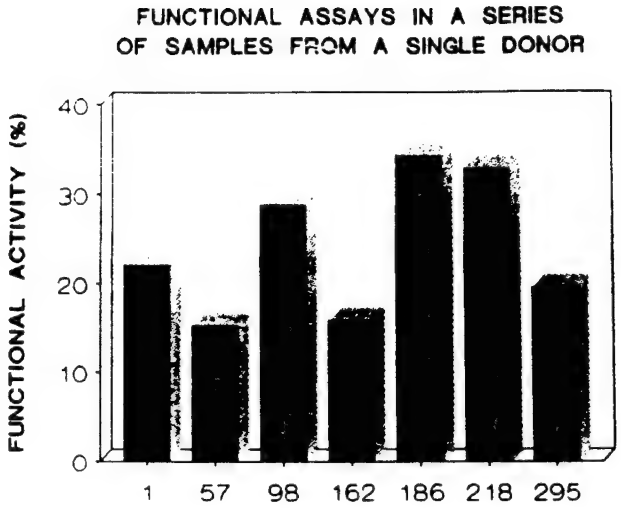
FIGURE 7a

The functional activity of fresh adherent mononuclear cells obtained from a single donor on 7 different occasions over a period of 295 days.

FIGURE 7b

The functional activity of fresh adherent mononuclear cells obtained from random donors on 10 different occasions over a period of 293 days.

FIGURE 7


FUNCTIONAL ASSAYS IN A SERIES
OF SAMPLES FROM A SINGLE DONOR
FUNCTIONAL ACTIVITY (%)
40
30
20
10
0
1
57
98
162
186
218
295

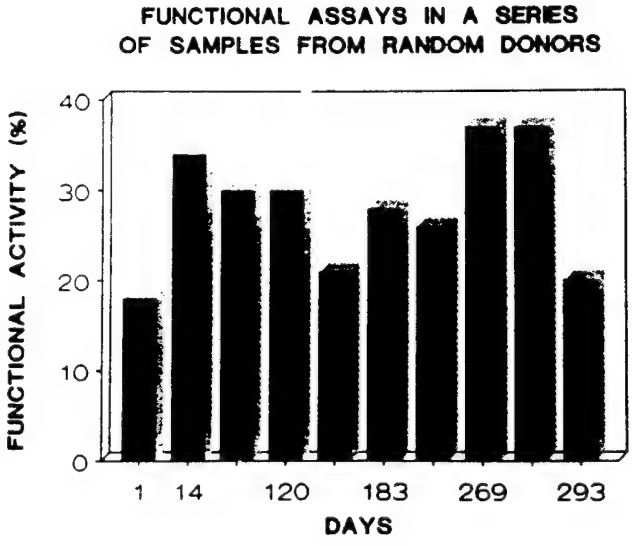

FUNCTIONAL ASSAYS IN A SERIES
OF SAMPLES FROM RANDOM DONORS
FUNCTIONAL ACTIVITY (%)
40
30
20
10
0
1
14
120
183
269
293
DAYS

TABLE 1

STUDY 1. THE EFFECTS OF NON-HEAT-INACTIVATED FETAL CALF SERUM ON THE FUNCTIONAL ASSAY OF FRESH ADHERENT MONONUCLEAR CELLS

	MONOCYTES (%)	MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)				
	AFTER PLATING	A. INCUBATION WITHOUT MONOCYTES		B. INCUBATION WITH MONOCYTES		B - A. FUNCTIONAL ACTIVITY
	ADHERENT CELLS	WITHOUT RBC-AB*	WITH RBC-AB	WITHOUT RBC-AB	WITH RBC-AB	WITH RBC-AB
1	33.0	23.2	29.7	26.5	41.3	11.6
2	16.6	23.2	29.7	25.8	47.1	17.4
3	.5	12.3	11.3	92.5	43.1	31.8
4	.2	84.8	51.0	86.8	68.3	17.3
5			41.2		79.5	38.3
6			56.2		82.5	26.3
7	77.5	88.6	86.4	86.1	73.9	0.0
8			82.0		67.7	0.0
9			87.9		80.5	0.0
10	79.0	77.8	53.5	80.8	46.7	0.0
11	39.5	62.8	17.1	71.8	38.4	21.3
12	70.7	62.8	17.1	66.5	35.5	18.4
13	21.1	47.9	22.7	54.8	30.7	8.0
14	78.9	97.0	76.5	91.6	65.5	0.0
15	78.3	96.7	79.3	90.3	73.9	0.0
16	43.0	61.1	53.4			
17		62.0	61.0			
18		66.0	66.0			
MEAN:	57.4	61.8	51.2	70.4	58.3	12.7
SD:	25.9	28.2	27.3	25.8	19.0	13.4
N:	12	14	18	12	15	15

*RBC-AB: RED BLOOD CELL ANTIBODY

TABLE 2

STUDY 1. THE EFFECTS OF THE PROPORTIONS OF ANTIBODY COATED RED BLOOD CELLS AND ADHERENT MONONUCLEAR CELLS (adMNC) ON THE FUNCTIONAL ASSAY USING HEAT-INACTIVATED FETAL CALF SERUM

MONOCYTE FUNCTIONAL ASSAY (% RBC 51Cr RELEASE)

A. INCREASING RED CELL CONCENTRATION

	FRESH ADHERENT MONONUCLEAR CELLS					CRYOPRESERVED ADHERENT MONONUCLEAR CELLS				
UNIT #	.25 RBC 1 adMNC	.5 RBC 1 adMNC	1 RBC 1 adMNC	2 RBC 1 adMNC	3 RBC 1 adMNC	.25 RBC 1 adMNC	.5 RBC 1 adMNC	1 RBC 1 adMNC	2 RBC 1 adMNC	3 RBC 1 adMNC
1	49.2	42.1	36.1	26.9	---	52.8	54.0	48.6	37.8	29.3
2	40.5	36.6	24.8	15.7	18.8	52.4	58.1	44.2	41.1	32.6
3	29.1	32.0	30.4	34.8	31.1					
MEAN:	39.6	36.9	30.4	25.8	25.0	52.6	56.1	46.4	39.5	31.0
SD:	8.2	4.1	4.6	7.8	6.2	0.2	2.1	2.2	1.7	1.7
N:	3	3	3	3	2	2	2	2	2	2

B. INCREASING ADHERENT MONONUCLEAR CELL CONCENTRATION

	FRESH ADHERENT MONONUCLEAR CELLS					CRYOPRESERVED ADHERENT MONONUCLEAR CELLS				
UNIT #	.25 adMNC 1 RBC	.5 adMNC 1 RBC	1 adMNC 1 RBC	2 adMNC 1 RBC	3 adMNC 1 RBC	.25 adMNC 1 RBC	.5 adMNC 1 RBC	1 adMNC 1 RBC	2 adMNC 1 RBC	3 adMNC 1 RBC
4	21.7	31.3	51.3	51.1	51.1	14.2	26.9	51.8	64.7	45.3
5	---	---	---	---	---	16.3	26.2	39.4	60.7	48.5
6	---	---	---	---	---	4.6	13.1	28.3	41.9	---
7	15.7	27.3	39.2	40.5	---	9.4	19.8	39.0	---	---
8	---	---	---	---	---	13.9	22.8	27.3	25.8	---
9	31.9	46.1	53.3	46.6	---	18.4	33.0	43.0	41.5	---
MEAN:	23.1	34.9	47.9	46.1	65.6	12.8	23.6	38.1	46.9	46.9
SD:	6.7	8.1	6.2	4.3	---	4.6	6.2	8.4	14.2	1.6
N:	3	3	3	3	1	6	6	6	2	2

TABLE 3.

STUDY 1. FUNCTIONAL ACTIVITY OF FRESH AND CRYOPRESERVED ADHERENT MONONUCLEAR CELLS USING HEAT-INACTIVATED FETAL CALF SERUM

	MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)								
	FRESH ADHERENT MONONUCLEAR CELLS					CRYOPRESERVED ADHERENT MONONUCLEAR CELLS				
	A. INCUBATION WITHOUT MONOCYTES		B. INCUBATION WITH MONOCYTES		B - A. FUNCTIONAL ACTIVITY	A. INCUBATION WITHOUT MONOCYTES		B. INCUBATION WITH MONOCYTES		B - A. FUNCTIONAL ACTIVITY
ID	WITHOUT RBC-AB*	WITH RBC-AB	WITHOUT RBC-AB	WITH RBC-AB	WITH RBC-AB	WITHOUT RBC-AB	WITH RBC-AB	WITHOUT RBC-AB	WITH RBC-AB	WITH RBC-AB
1	2.6	3.6	3.1	31.4	27.8	4.5	3.4	3.1	60.9	57.5
1						5.2	3.4	5.9	51.4	48.0
2	2.3	3.1	3.1	12.9	9.8	4.5	3.4	2.7	51.9	49.8
3	2.4	2.5	2.5	54.1	51.6	4.0	6.9	5.2	42.8	49.8
4		2.6		48.9	46.1	5.9	4.8	5.9	51.4	46.6
4							3.1		53.4	50.3
5	2.8	2.6	3.6	45.2	42.6					
6						8.5	9.3	2.9	56.7	47.2
7	5.2	3.2	3.8	43.0	39.8	8.5	9.4	3.2	48.9	39.5
7						5.9	4.7	6.0	44.5	39.7
7						5.9	4.8	4.0	41.3	36.6
7						4.1	3.8	6.0	59.5	55.7
8	2.4	3.4	3.5	64.6	61.2					
9	6.0	5.8	4.3	56.6	50.8					
10	4.1	4.9	4.0	45.9	41.0	4.1	3.9	6.3	54.3	50.4
11	4.5	3.8	6.3	45.9	42.0	3.3	3.2	3.9	29.4	26.2
12	8.0	3.2	3.0	33.5	30.2					
						5.4	4.9	4.6	49.7	44.8
MEAN	4.0	3.5	3.7	43.8	40.3	1.7	2.2	1.4	8.5	8.9
SD	1.9	1.0	1.1	14.0	13.8	12	13	12	13	13
N	10	11	10	11	11					

*RBC-AB: RED BLOOD CELL ANTIBODY

TABLE 4.

STUDY 1. THE FUNCTIONAL ACTIVITY OF FRESH AND CRYOPRESERVED ADHERENT MONONUCLEAR CELLS USING HEAT-INACTIVATED FETAL CALF SERUM.

	MONOCYTES (%)	MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)				
		A. INCUBATION WITHOUT MONOCYTES		B. INCUBATION WITH MONOCYTES		B - A. FUNCTIONAL ACTIVITY
	AFTER PLATING					
	ADHERENT CELLS	WITHOUT RBC-AB*	WITH RBC-AB	WITHOUT RBC-AB	WITH RBC-AB	WITH RBC-AB
FRESH						
MEAN	60.9	4.0	3.5	3.7	43.8	40.3
SD	22.5	1.9	1.0	1.1	14.0	13.8
N	11.0	10.0	11.0	10.0	11.0	11.0
RANGE	14-90	2-8	2-6	2-6	13-65	10-61
CRYOPRESERVED						
MEAN	42.2	5.4	4.9	4.6	49.7	44.8
SD	15.7	1.7	2.2	1.4	8.5	8.9
N	13.0	12.0	13.0	12.0	13.0	13.0
RANGE	13-67	3-9	3-9	2-6	30-61	26-58

*RBC-AB: RED BLOOD CELL ANTIBODY

TABLE 5.

STUDY 2. VIABILITY AND FUNCTIONAL ACTIVITY OF FRESH MONONUCLEAR CELLS USING HEAT-INACTIVATED FETAL CALF SERUM.

	VIABILITY (%)		MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)				
	BEFORE PLATING	AFTER PLATING	A. INCUBATION WITHOUT MONOCYTES		B. INCUBATION WITH MONOCYTES		B - A. FUNCTIONAL ACTIVITY
	ALL CELLS	ADHERENT CELLS	WITHOUT RBC-AB*	WITH RBC-AB	WITHOUT RBC-AB	WITH RBC-AB	WITH RBC-AB
1	95	90	5.2	5.6	5.2	46.9	41.3
2	100	92	4.1	4.0	4.1	26.1	22.1
3	98	83	4.1	3.6	4.1	21.6	18.0
4	98	94	4.9	4.7	8.5	38.7	34.0
5	99	75	2.8	2.6	3.6	23.6	21.0
6	99	81	6.4	3.4	6.4	43.1	39.7
7	97	76	3.3	3.1	4.6	14.8	11.7
8	100		3.2	2.6	4.5	7.3	4.7
9	100	92	3.0	3.0	3.6	18.3	15.3
10	94	98	8.9	10.2	8.9	40.3	30.1
11	99		10.5	4.7	10.5	41.5	36.8
12	94	99	13.1	10.8	22.2	39.7	28.9
13	99		11.7	14.5	11.7	57.8	43.3
14	100		3.8	2.5	5.9	32.1	29.6
15	99	66	2.9	3.3	3.5	31.7	28.4
16	98	79	2.5	2.9	2.5	46.4	43.5
17	99	94	2.4	2.7	3.3	34.8	32.1
18	99		2.6	2.0	2.6	23.1	21.1
19	99		2.5	2.7	2.6	18.6	15.9
20	99	98	3.5	2.9	4.3	32.1	29.2
21	100	91	2.8	2.5	2.8	34.8	32.3
22	94	87	3.1	2.2	3.1	31.9	29.7
23	99	97	5.2	2.7	5.2	37.0	34.3
24	99	87	2.7	2.4	3.1	30.4	28.0
25	99	93	4.5	2.2	4.5	25.9	23.7
26	99	85	3.1	3.6	3.1	41.0	37.4
27	99	96	2.7	3.1	2.8	36.9	33.8
28	99	91	3.0	2.9	3.0	35.8	32.9
29	100	94	2.5	2.9	2.5	25.9	23.0
30	100	100	5.7	3.8	5.7	29.7	25.9
31	98	97	4.4	2.8	4.9	39.4	36.6
32	97	87	5.6	3.9	5.6	37.7	33.8
33	95	90	4.1	3.3	4.1	30.4	27.1
34	99	98	4.0	4.2	4.0	40.7	36.5
35	99	98	9.3	6.6	9.3	26.2	19.6
MEAN	98	90	4.7	4.0	5.3	32.6	28.6
SD	2	8	2.8	2.7	3.8	10.3	9.2
N	35	29	35	35	35	35	35

*RBC-AB: RED BLOOD CELL ANTIBODY

TABLE 6

STUDY 2. VIABILITY AND FUNCTIONAL ACTIVITY OF CRYOPRESERVED MONONUCLEAR CELLS USING HEAT-INACTIVATED FETAL CALF SERUM.

		VIABILITY (%)		MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)				
				A. INCUBATION WITHOUT MONOCYTES		B. INCUBATION WITH MONOCYTES		B - A. FUNCTIONAL ACTIVITY
	STORAGE AT -80C (DAYS)	BEFORE PLATING	AFTER PLATING					
		ALL CELLS	ADHERENT CELLS	WITHOUT RBC-AB	WITH RBC-AB*	WITHOUT RBC-AB	WITH RBC-AB	WITH RBC-AB
	79	98	96	11.7	14.5	8	50.3	35.8
	82	87	89	3.4	2.2	3.9	17.6	15.4
	98	94	95	2.5	2.9	2.7	34.5	31.6
	100	87	84	13.1	10.8	14.1	37.7	26.9
	113	75		2.5	2.7	2.7	3.4	0.7
	118	91	75	4.1	3.3	6.5	30.1	26.8
	153	91	83	4.4	2.8	4.5	38.7	35.9
	175	95	97	2.8	2.5	2.8	36.3	33.8
	232	87	86	5.6	3.9	5.7	5.6	1.7
	418	91	87	5.2	2.7	5.2	15.9	13.2
	936	97	93	5.7	3.8	8.9	28.8	25
MEAN	228	90	89	5.5	4.7	5.9	27.2	22.4
SD	267	7	7	3.8	4.2	3.6	15.5	13.5
N	11	11	10	11	11	11	11	11

*RBC-AB: RED BLOOD CELL ANTIBODY

TABLE 7.

THE FUNCTIONAL ACTIVITY OF FRESH AND CRYOPRESERVED ADHERENT MONONUCLEAR CELLS USING HEAT-INACTIVATED FETAL CALF SERUM IN STUDIES 1 AND 2.

	MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)			
	FRESH adMNC*	CRYOPRESERVED adMNC	FRESH CONTROLS ASSAYED WITH CRYOPRESERVED adMNC	CRYOPRESERVED adMNC (% OF FRESH CONTROL)
STUDY 1: -80C STORAGE FOR AS LONG AS 126 DAYS				
MEAN	40.3	44.8	-	-
SD	13.8	8.9		
RANGE	10-61	26-58		
N	11	13		
STUDY 2: -80C STORAGE FOR AS LONG AS 936 DAYS				
MEAN	27.3	22.4 **	31.9	68
SD	10.3	12.3	7.5	36
RANGE	3.5-44	0.7-36	16-44	4-105
N	37	11	11	11

*adMNC: adherent mononuclear cells

**paired t test: $p<0.02$, cryopreserved vs fresh control

TABLE 8.

STUDY 2. FUNCTIONAL ACTIVITY OF ADHERENT MONONUCLEAR CELLS (adMNC) BEFORE AND AFTER CRYOPRESERVATION

		MONOCYTE FUNCTIONAL ASSAY (% RBC 51CR RELEASE)			
	STORAGE AT -80C (DAYS)	adMNC BEFORE FREEZING	adMNC AFTER FREEZING	FRESH CONTROLS ASSAYED WITH CRYOPRESERVED adMNC	adMNC AFTER FREEZING (% OF CONTROL)
	79	39.7	35.8	43.3 *	82.7
	82	28.9	15.4	29.7	51.9
	100	22.1	26.9	28.9 *	93.1
	113	15.3	0.7	15.9 *	4.4
	118	21.1	26.8	27.1 *	98.9
	175	18.0	33.8	32.3 *	104.6
	232	11.7	1.7	33.8 *	5.0
MEAN	128	22.4	20.2	30.1	62.9
SD	61	10.2	15.6	8.9	46.8
N	7	7	7	7	7

*FRESH CONTROL MONOCYTES FROM SAME SUBJECT AS CRYOPRESERVED MONOCYTES

**PAIRED T TEST: $p<0.02$, CRYOPRESERVED vs FRESH CONTROL

TABLE 9.

STUDY 2. PERCENTAGE OF MONOCYTES IDENTIFIED BY VOLUME DISTRIBUTION IN MONONUCLEAR CELL SAMPLES BEFORE PLATING AND IN ADHERENT MONONUCLEAR CELL SAMPLES AFTER PLATING.

	% MONOCYTES			
	% PRIOR TO CRYOPRESERVATION		% FOLLOWING CRYOPRESERVATION	
UNIT NO.	BEFORE PLATING	AFTER PLATING	BEFORE PLATING	AFTER PLATING
1	33	63	18	30
2	35	63	30	80
3	25	52	26	16
4			54	73
5			26	17
6			23	20
7			11	21
8			28	17
9			34	29
10	24	40		
11	21	65		
12	29	67		
13	19	58		
14	10	63		
15	17	43		
16	20	62		
17	17	55		
18	54	72		
19	54	73		
20	28	14		
21	13	12		
22	34	47		
23	15	55		
24	55	32		
25	21	55		
26	27	29		
27	15	26		
28	17	13		
29	17	10		
30	33	63		
31	18	12		
32	21	36		
33	18	45		
MEAN	25	45	28	34
SD	13	21	13	26
N	27	27	9	9
PAIRED t: p= (PRE-POST PLATE)	<0.001		NS	

STUDY 2 TABLE 10

FUNCTIONAL ACTIVITY OF FRESH ADHERENT MONONUCLEAR CELLS CORRECTED FOR %VIABILITY AND THE %MONOCYTES.

	A	B	C = A X B	D	E = D / C
	VIABILITY (%)	MONOCYTES (%)	VIABLE MONOCYTES (%)	FUNCTIONAL ASSAY (%)	CORRECTED FUNCTIONAL ASSAY (%)
1	90	65	58	41	71
2	92	63	58	22	38
4	94	67	63	34	54
6	81	63	51	40	78
7	76	72	55	12	21
9	92	58	53	15	29
10	98	63	62	30	49
12	99	52	51	29	56
15	66	72	48	28	60
16	79	73	58	44	75
17	94	14	13	32	244
20	98	12	12	29	248
21	91	47	43	32	76
22	87	55	48	30	62
23	97	32	31	34	111
26	85	55	47	37	80
27	96	29	28	34	121
28	91	26	24	33	139
29	94	13	12	23	188
30	100	10	10	26	259
32	87	63	55	34	62
33	90	12	11	27	251
34	98	36	35	37	103
35	98	45	44	20	44
MEAN	91	46	40	30	105
SD	8	22	19	8	77
N	24	24	24	24	24

TABLE 11

STUDY 2.
FUNCTIONAL ASSAYS OF FRESH ADHERENT MONONUCLEAR CELLS IN SAMPLES COLLECTED FROM ONE DONOR OVER A PERIOD OF 295 DAYS, AND FROM A SECOND DONOR OVER A PERIOD OF 267 DAYS.

DONOR	SAMPLE	DAY	FUNCTIONAL ASSAY (% 51CR RELEASE FROM ANTIBODY-COATED RBC)
1	1	1	22.1
1	2	57	15.3
1	3	98	28.9
1	4	162	15.9
1	5	186	34.3
1	6	218	32.9
1	7	295	19.6
2	1	1	18.0
2	2	34	11.7
2	3	146	28.4
2	4	176	32.3
2	5	204	37.4
2	6	267	33.8